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TECHNICAL MANUSCRIPT 419

EVIDENCE THAT TWO MAJOR REPLICONS COMPRISE THE GENOME OF STAPHYLOCOCCUS AUREUS

Robert A. Altenbern

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DECEMBER 1967

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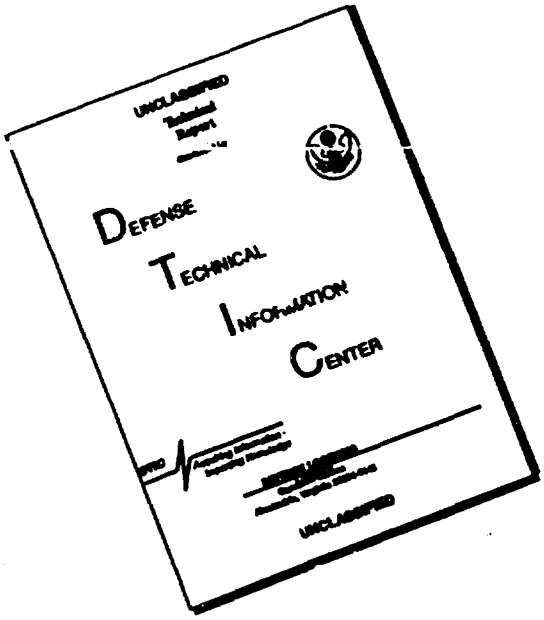

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TECHNICAL MANUSCRIPT 419

EVIDENCE THAT TWO MAJOR REPLICONS COMPRISE THE GENOME OF *STAPHYLOCOCCUS AUREUS*

Robert A. Altenbern

Medical Bacteriology Division
BIOLOGICAL SCIENCES LABORATORY

Project 1C014501B71A

December 1967

ABSTRACT

In *Staphylococcus aureus*, a pronounced shift in position of the acriflavin resistance locus was observed when gene order was determined by marker frequency analysis of cells of various ages. In young cells (2-hour culture), acriflavin resistance was mapped near the origin of replication, but, in older cells (5-hour culture), the same locus was mapped at the middle of the genome. By the method of synchronous chromosomal replication, acriflavin resistance consistently duplicated in the central portion of the chromosome. When cells from a 2-hour culture were exposed to nitrosoguanidine and then allowed to express mutations after mutagen treatment, novobiocin-resistant mutants were fully expressed after two generations, but acriflavin-resistant mutants were not fully expressed until after five to six generations. In contrast, when cells from a 5-hour culture were similarly treated, both novobiocin resistance and acriflavin resistance were fully expressed after two generations of growth. Furthermore, the ratio of frequency of acriflavin-resistant mutants to novobiocin-resistant mutants was 2.5 times higher in 2-hour cells than in 5-hour cells.

When cells of various ages were exposed to phenethyl alcohol for 2 hours before exposure to nitrosoguanidine, some genes showed a constant ratio between the frequency of induced mutants compared with the frequency of induced mutations to novobiocin resistance, regardless of the age of the cells. Other genes, however, exhibited a much higher ratio of frequency compared with novobiocin resistance in 2-hour cells than in 5-hour cells.

These data have been utilized to demonstrate that the total genome in *S. aureus* is composed of two replicons. In young cells, one replicon occurs in a much greater number of copies compared with other replicon, but this imbalance of replicon ratio declines considerably in older cells. Nine genes have been examined to date. Three of these genes have been assigned to one replicon, and the other six to the second replicon.

I. INTRODUCTION

Many reports have given firm evidence that the genome of *Escherichia coli*, and probably other enterics, exists as a single but circular DNA molecule constituting a single replicon.[1] Evidence has accumulated[2] that the chromosome of *Bacillus subtilis* also exists as a single DNA molecule and behaves as a continuous linkage group within the confines of the replication origin and terminus. Investigations in this laboratory have led to a method for mapping the chromosome of *Staphylococcus aureus* by synchronizing chromosomal replication. Gene frequency analysis methodology developed by Sueoka and associates has been applied to *S. aureus* and supports the genomic map obtained by the synchronous chromosomal replication method. However, certain anomalies arose during derivation of a chromosomal map by gene frequency analysis that were inconsistent with the concept of a single replicon for the total genome of *S. aureus*. This report presents evidence that the genome of this organism is composed of two major replicons, one of which is present in a significantly greater number of copies than the other during early log phase.

II. MATERIALS AND METHODS

The strain of *S. aureus* employed in this study was isolated from clinical material. It was maintained on trypticase soy (TS) agar* slants and stored at 4 C. Transfers were made every 3 months. Singly auxotrophic mutants of this strain were isolated following exposure of the wild type to nitrosoguanidine. All broth media were TS broth. Minimal agar was a hydrolyzed casein medium whose composition was described previously.[3]

The synchronous chromosomal replication method of genomic mapping has been published.[3] Gene frequency analysis methodology for *S. aureus* gene order is described elsewhere** and is similar to the method of Yoshikawa and Sueoka.[4]

Broth cultures (5 ml per tube) were inoculated with 0.1 ml of a 1:10 dilution of an overnight culture in TS broth and incubated on a shaker at 37 C. After 2, 3, 4, and 5 hours of incubation, the optical densities at 650 mμ were 0.06, 0.25, 0.48, and 0.75, respectively, for the wild type. Some of the auxotrophic mutants exhibited a slower rate of growth than that of the parent type.

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* Baltimore Biological Laboratories.
** Altenbern, R.A., unpublished data.

Plating medium for detection of inhibitor-resistant mutants was TS agar containing any one of the following inhibitors: chloramphenicol, 4 μg/ml; novobiocin, 2 μg/ml; nitrofurazone, 8 μg/ml; acriflavin, 25 μg/ml. Mutations to nonpigmentation were detected by plating on TS agar and incubating for 24 hours at 37 C followed by an additional 24-hour incubation at room temperature to intensify pigmentation of the wild type and to enhance contrast between pigmented and nonpigmented clones. All platings were performed in triplicate or quadruplicate. Mutant frequency ratios and gene frequency analysis were established from total mutant count on triplicate or quadruplicate plates of a specific dilution. When necessary, total viable count was determined by triplicate platings on TS agar.

III. RESULTS

The locations of the genes considered here are presented in Figure 1, as determined by the synchronous chromosomal replication technique in which chromosomal duplication requires 120 minutes. It should be noted that the acriflavin resistance locus duplicates at the same time as the novobiocin resistance locus. Gene frequency experiments designed to show forking of chromosomal replication during various stages of logarithmic growth indicated that considerable forking did occur during early log phase (Table 1). The gene controlling acriflavin resistance had apparently shifted position, as evidenced by comparing gene order of 2-hour cells with that of 5-hour cells. Neither reversal of polarity of chromosomal replication nor extensive forking during genomic duplication can satisfactorily explain the shift of position of the acriflavin resistance gene. This marker behaves as a single locus with a chromosomal position defined as shown in Figure 1. The concept was then developed that the acriflavin resistance locus occurred on a replicon separate from the replicon bearing the other genes presented in Table 1. It would be necessary for the replicon bearing acriflavin resistance (replicon I) to be present in a greater number of copies in early log phase (2-hour cultures) than the replicon (replicon II) containing the other genes in Table 1. Furthermore, in mid to late log phase cultures (5 hours), the number of copies of replicon I compared with replicon II should decline several fold. This hypothesis would explain satisfactorily the shifting position of the acriflavin resistance gene compared with the other genes as a function of culture age.

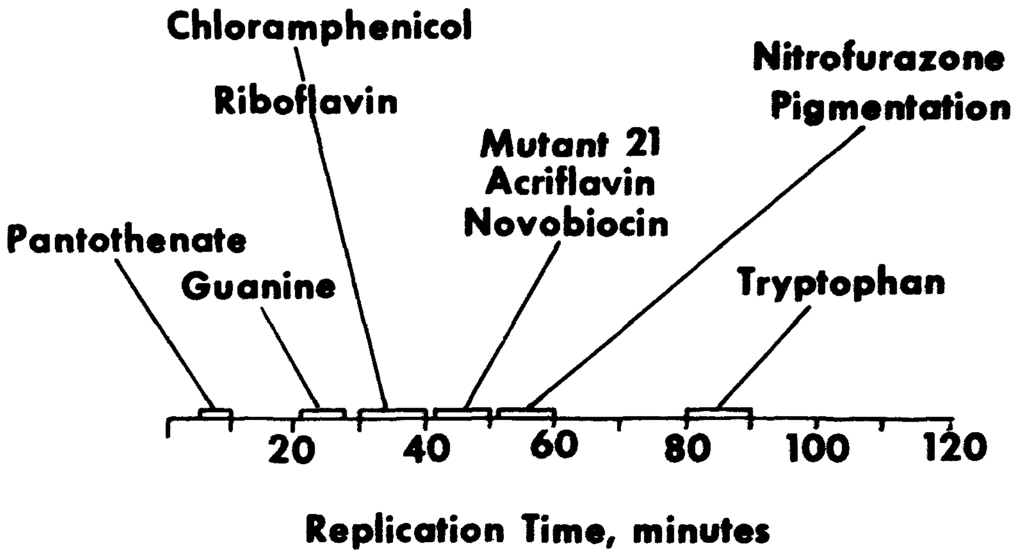

Figure 1. Chromosome Map of Staphylococcus aureus, Strain Maybush.

TABLE 1. GENE ORDER[a/] ON STAPHYLOCOCCUS AUREUS CHROMOSOME OBTAINED BY GENE FREQUENCY ANALYSIS ON CELLS OF DIFFERENT AGES

Loci[b/]	Age of Cells, hours: 2	4	5	6
ACR/NOV	5.8	1.3	0.68	0.42
ACR/NFN	6.37	1.36	2.0	2.54
CMP/NOV	3.0	1.94	1.05	1.39

a. Gene order at 2 hours: ACR-CMP-NOV-NFN.
Gene order at 5 hours: CMP-NOV-ACR-NFN.

b. ACR = acriflavin resistance; NOV = novobiocin resistance; NFN = nitrofurazone resistance; CMP = chloramphenicol resistance.

The hypothesis was tested by examining the kinetics of expression of acriflavin resistance (on replicon I) compared with expression of novobiocin resistance (on replicon II). Two-hour and 5-hour cultures were prepared as indicated in Section II. The 5-hour culture was diluted 1:10 in fresh medium so that both cultures possessed similar optical densities (0.05 to 0.06 at 650 mμ). Equal volumes (5 ml) of these cells were then centrifuged and resuspended in equal volumes of saline containing 200 μg of nitrosoguanidine per ml. After exposure to this mutagen for 20 minutes at 30 C, the cells were recovered by centrifugation and resuspended in 5 ml of fresh, prewarmed (37 C) TS broth and placed on a shaker at 37 C. At time zero and at appropriate intervals thereafter, each culture was diluted and plated for total viability count and for numbers of induced mutants to acriflavin resistance and to novobiocin resistance. The results are presented in Figure 2. It is clear that, with 2-hour cells, the expression of acriflavin resistance is significantly delayed as compared with expression of novobiocin resistance. In contrast, with 5-hour cells, the full expression of resistance to acriflavin occurs simultaneously with expression of novobiocin resistance. The ratio of frequency of acriflavin-resistant mutants to frequency of novobiocin-resistant mutants is 2.5 times higher with the 2-hour cells than with the 5-hour cells. These data are entirely consistent with the hypothesis that there are many more copies of replicon I than of replicon II at 2 hours. Such a situation would result in a long segregation lag for expression of acriflavin resistance compared with novobiocin resistance and would yield a much greater relative number of acriflavin-resistant mutants than novobiocin-resistant mutants because there would be so many more copies of the acriflavin resistance locus. In 5-hour cells, where duplication of replicon I compared with duplication of replicon II has hypothetically diminished, it would be expected that expression kinetics for acriflavin resistance and for novobiocin resistance would be approximately similar. In addition, it could be predicted that the ratio of frequency of acriflavin mutants to frequency of novobiocin mutants would decline because of the smaller number of copies of replicon I compared with replicon II.

Although the experiments outlined above could be applied to the specific case where the acriflavin locus duplicated at the same time as the novobiocin locus on the genomic map, the argument loses its validity when directed toward a locus that duplicates <u>earlier</u> than that of novobiocin resistance. In such a case, delayed expression and greater numbers of mutants of the early gene than at the novobiocin locus for 2-hour cells compared with 5-hour cells might well be interpreted as evidence of more extensive forking during chromosomal duplication in young cells versus older cells.

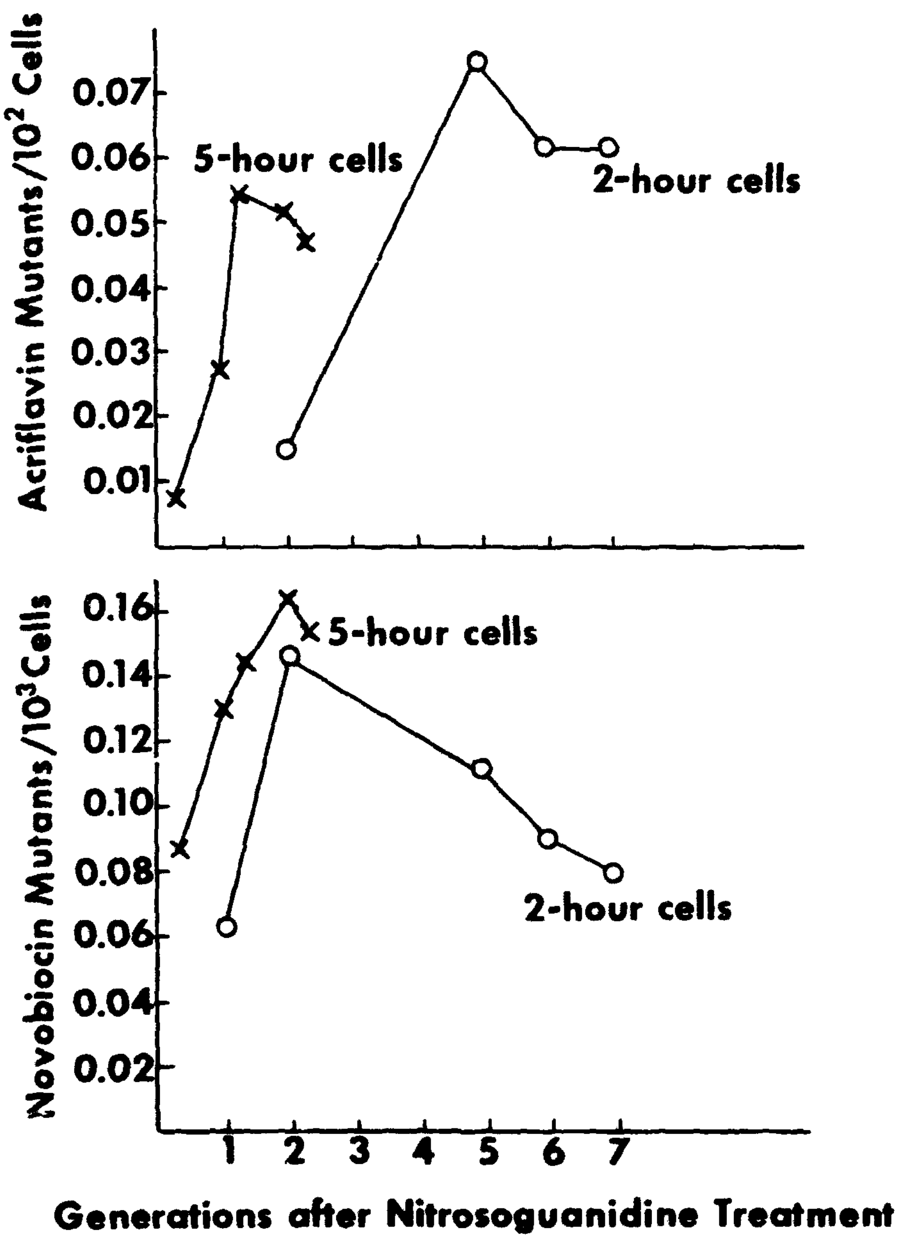

Figure 2. Expression of Mutations to Acriflavin Resistance and to Novobiocin Resistance in 2-Hour and 5-Hour Cells after Exposure to Nitrosoguanidine.

Therefore, a technique was adopted whereby the numerical ratios of the number of copies of replicon I compared with replicon II could be "frozen." This was done by adding 0.40% phenethyl alcohol (PEA) to the cultures and incubating them at 30 C for 2 hours. It has been shown[3,4] that this treatment allows chromosomal replication to proceed but prevents any subsequent reinitiation of duplication of the genome. Cells so treated would be held invariant regarding the number of copies of both replicon I and II. Cultures of the wild type and the various auxotrophic mutants were grown in TS broth for 2, 3, 4, and 5 hours. PEA was then added to a final concentration of 0.40% (v/v). The cultures were incubated for 2 hours at 30 C to allow replication in progress to proceed to completion. The 3-, 4-, and 5-hour cultures were diluted 1:2, 1:4, and 1:10, respectively, in TS broth containing 0.40% PEA to equal approximately the optical density of the 2-hour culture (0.05 to 0.06). The cells from equal volumes of culture were then removed by centrifugation and resuspended in 5 ml of saline containing 200 µg of nitrosoguanidine per ml. After 20 minutes of incubation at 30 C, the cells were recovered by centrifugation and resuspended in 5 ml of fresh TS broth. The resulting cultures were incubated on a shaker at 37 C for 5 hours to insure full expression of all mutations. The cells were then diluted and plated both on TS agar containing 2 µg of novobiocin per ml and on either TS agar containing another appropriate inhibitor or minimal agar to score for revertants to prototrophy. The novobiocin resistance locus was employed as a reference marker because general experience had shown that this gene could be consistently scored with high accuracy. The ratio of the number of mutants at a specific locus compared with the number of novobiocin-resistant mutants was calculated for the 2-, 3-, 4-, and 5-hour cells. The results of four such determinations are presented in Fig. 3. Note that for both the pantothenate locus and the acriflavin resistance locus, the ratio of mutants is high compared with novobiocin-resistant mutants for 2-hour cells but declines rapidly for 3-, 4-, and 5-hour cells. These results are in sharp contrast with results obtained at both the tryptophan locus and the nitrofurazone resistance locus, where the ratios of mutants compared with novobiocin-resistant mutants are essentially constant. On the basis of such data, loci for acriflavin resistance and pantothenate are assigned to replicon I, and loci for novobiocin resistance and tryptophan can be assigned to replicon II. The pantothenateless mutant grows noticeably more slowly in TS broth than most of the other mutants or the wild type, so that the decline in ratio for pantothenate to novobiocin is less precipitous than that for acriflavin to novobiocin. Table 2 compares the ratios of the frequency of mutants at a variety of loci (Fig. 1) to the frequency of novobiocin-resistant mutants. Although some loci show a slightly erratic ratio of frequencies, the two types of relationship can be clearly detected as depicted in Figure 3. Mutant 21 is an auxotrophic mutation, not yet defined, that has a gene duplication time of 40 to 50 minutes on the 120-minute map (Fig. 1).

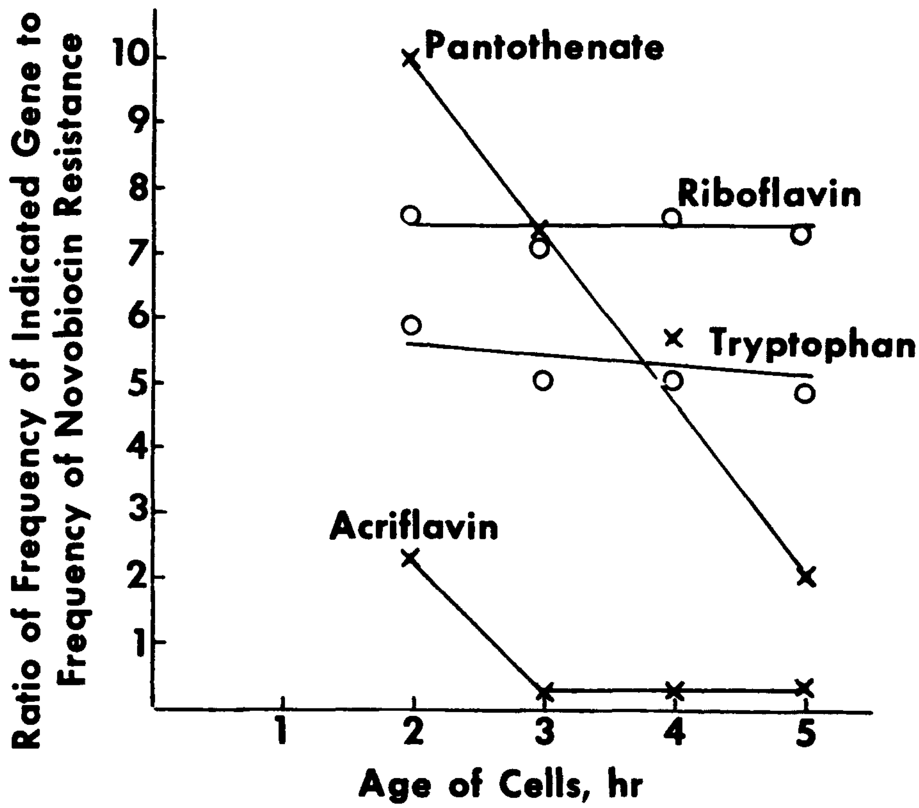

Figure 3. Ratios of Mutations of Several Loci to Mutations to Novobiocin Resistance in 2-, 3-, 4- and 5-Hour Cells.

TABLE 2. RATIOS OF FREQUENCY OF SINGLE GENETIC LOCI TO NOVOBIOCIN RESISTANCE LOCUS AS RELATED TO AGE OF CELLS

	Culture Age, hours			
Locus	2	3	4	5
ACR	2.2	0.35	0.25	0.41
NFN	2.1	2.5	1.8	2.6
CMP	1.9	1.5	1.3	1.7
PAN	10.0	7.4	5.7	2.0
RIB	0.77	0.72	0.75	0.67
GUA	0.04	0.024	0.027	0.023
TRY	6.0	5.1	5.1	4.8
MUT 21	5.1	1.3	0.6	0.6

Figure 4 presents the two hypothetical replicons bearing the respective genes at the duplication times determined by the synchronous chromosomal replication method. At present there is no distinctive arrangement of nutritional loci on one replicon and inhibitor resistance loci on the other; the distribution of genes appears to be random. It should be emphasized that no rational conclusion can be drawn concerning the absolute numbers of replicons per cell. The ratios tell only that at 2 hours, replicon I is 5.1 (mutant 21) to 6.4 (pantothenate) times as frequent as replicon II when compared with the ratio of frequencies in the 5-hour cells. This concept also explains an anomaly in gene frequency analysis concerning the normalized value of the ratio of the pantothenate gene to the novobiocin resistance gene, which was 12.0 or more in several determinations and appeared to be in error for unknown reasons. The existence of pantothenate and novobiocin resistance on separate replicons, one of which occurs in a greater number of copies than the other, would yield such high values.

No conclusions can be drawn concerning the relative size of the two replicons proposed for S. aureus. They seem to be of roughly equivalent lengths because replicon I bears a gene (acriflavin resistance) that duplicates at 40 to 50 minutes on the 120-minute map, but replicon II has only two later genes: nitrofurazone resistance (50 to 60 minutes) and tryptophan (80 to 90 minutes). The lack of resistance to the inhibitors in the wild type seems to eliminate the possibility of a replicon of the nature of RTF associated with penicillin resistance and erythromycin resistance as well as other antibiotics.[8]

In conclusion, this rationale and methodology has not been applied to other organisms. To the author's knowledge, there are no recorded cases of shifting gene order by gene frequency analysis in other bacteria as a function of the age of the cells. Although the concept of more than one replicon constituting the genome of a bacterium is novel, it is invariably true in higher organisms with visible chromosomes.

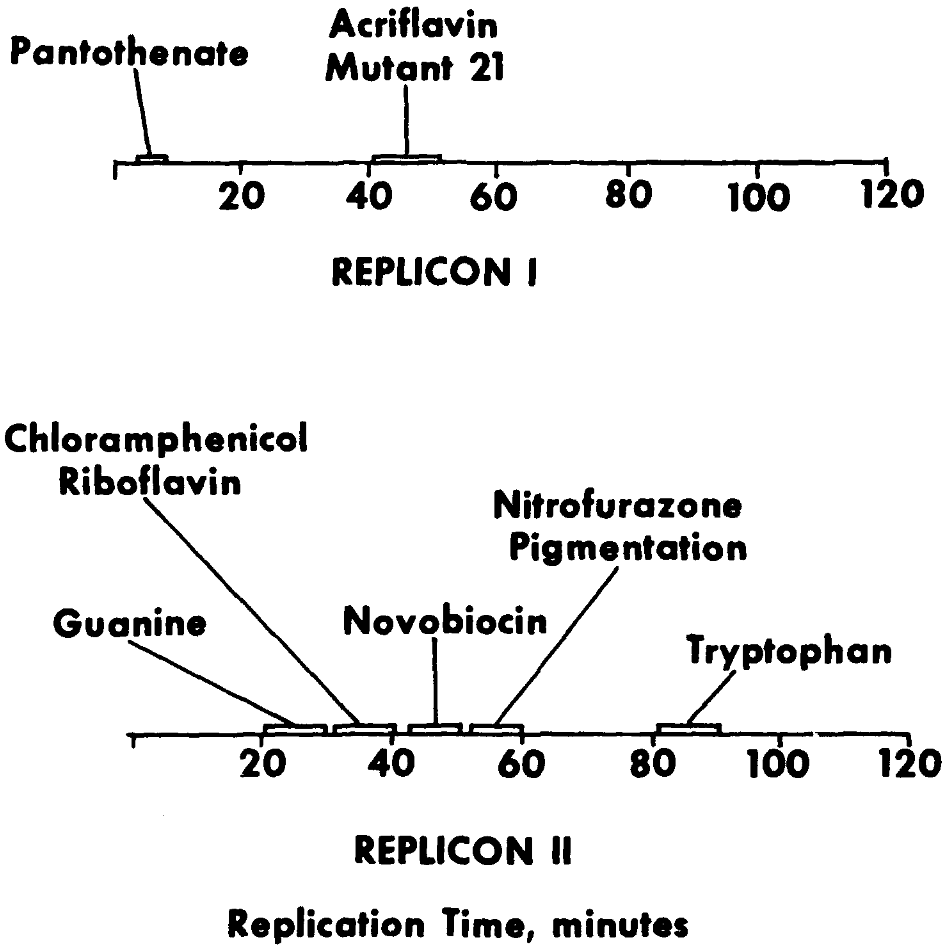

Figure 4. Two Hypothetical Replicons in Staphylococcus aureus. Pigmentation gene exhibits full expression of mutation after two generations for both 2-hour and 5-hour cells.

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Staphylococcus aureus						
Acriflavin						
*Replication						
Nitrosoguanidine						
*Mutations						
Novobiocin						

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